Supporting Information for:

Cellobiose Dehydrogenase and a Copper Dependent Polysaccharide Monooxygenase Potentiate Cellulose Degradation by *Neurospora crassa*

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SUPPLEMENTAL MATERIALS AND METHODS

Strains. All *Neurospora crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC) including wild-type *N. crassa* (FGSC 2489) and Δ*mus-51* (FGSC 9717).

Protein gel electrophoresis and quantification. Samples were mixed with SDS Laemmli loading buffer, boiled for 5 minutes, and loaded onto a Criterion 4-15% Tris-HCl polyacrylamide gel (Biorad). Gelcode Blue stain reagent (Thermo Scientific) was used for staining. Total extracellular protein content was determined using the Biorad Protein Assay (Biorad) according to the manufacturer's instructions.

Cellulase assays on Avicel®. Cellulase assays were performed in triplicate with 10 mg/mL Avicel® PH101 in 50 mM sodium acetate pH 5.0 at 40 °C. Assays were performed in 1.7 mL microcentrifuge tubes with 1.0 mL total volume and were inverted 20 times per minute. Each assay contained 0.05 mg/mL culture filtrate or 0.05 mg/mL reconstituted cellulase mixture containing CBH-1, GH6-2, GH5-1, and GH3-4 present in a ratio of 6:2.5:1:0.5(*1*). CDH addition to assays was at a concentration of 0.004 mg/mL unless otherwise noted. CDH-2 flavin domain was added on an equal activity basis relative to full-length CDH-2 as measured by the cellobiose dependent reduction of DCPIP.

To measure the percent degradation, Avicel® assays were centrifuged, and 20 µL of assay mix was removed per well. Samples were incubated with 100 µL of desalted, diluted Novozyme 188 (Sigma) at 40 °C for 20 minutes to hydrolyze cellobiose and then 10-30 µL of the Novozyme 188 treated cellulase assay supernatant was analyzed for glucose using the glucose oxidase/peroxidase assay as described previously (*2*). Percent

degradation was calculated based on the maximum theoretical conversion of 10 mg/mL Avicel®.

Phylogeny of *N. crassa* PMO proteins. Amino acid sequences were processed to remove signal peptides so that every sequence started with histidine 1. The C-terminus of each protein was also truncated to remove linkers or cellulose binding modules present on some PMOs. Multiple sequence alignments were performed locally using T-COFFEE (*3*). A maximum likelihood phylogeny was determined using PhyML version 3.0 with 500 bootstraps through the Phylogeny.fr webserver (*4*).

Protein purification. For isolation of *Myceliophthora thermophila* (ATCC 46424) CDH-1 and CDH-2, 10 day old conidia from cultures of *M. thermophila* were inoculated into 1.0 liter of Vogel's salts supplemented with 40 g/L cotton balls in 2.8 liter Fernbach flasks. After 6 days of growth at 48 °C and 200 RPM shaking, fungal biomass and residual cotton was removed by filtration using 0.2 µm PES filters. The culture filtrate was then passed over CaptoQ resin (GE Healthcare) at 20 mL/min. CDH-1 and CDH-2 were eluted from the CaptoQ resin with buffer containing 500 mM sodium chloride and 25 mM HEPES pH 7.4. The pooled CaptoQ concentrate was then desalted into 25 mM HEPES pH 7.4 using a HiPrep 26/10 desalting column (GE Healthcare). The protein was further purified on a 10/100 GL MonoQ column (GE Healthcare) and eluted with a linear gradient from 0 to 500 mM sodium chloride. CDH-1 elutes from the column between 180 and 220 mM sodium chloride; CDH-2 elutes between 280 and 320 mM sodium chloride. CDH-1 was then further purified by binding to 1.0 grams of Avicel® in 50 mM sodium acetate buffer pH 5.0 for 5 minutes at 50 °C. The Avicel® was removed by centrifugation in 50 mL conical tubes then washed with 50 mL of acetate buffer three

times. CDH-1 was eluted from the Avicel® by addition of 50 mL of 50 mM pH 11.3 phosphate buffer. The eluted CDH-1 was concentrated using 10,000 molecular weight cutoff (MWCO) PES spin concentrators and stored at -80 °C. CDH-2 was further purified by gel filtration chromatography using a 16/60 Sephacryl S200 column (GE Healthcare) with a mobile phase of 25 mM HEPES pH 7.4 and 150 mM sodium chloride. The flavin domain of CDH-2 was isolated by treatment of the full length enzyme with papain (*5*). After papain cleavage, the fragments were separated by gel filtration chromatography using a 16/60 Sephacryl S100 column (GE Healthcare) with a mobile phase of 25 mM HEPES pH 7.4 and 150 mM sodium chloride. The fractions enriched in flavin domain were then further purified using a 10/100 GL MonoQ column and eluted with a linear gradient from 0 to 500 mM sodium chloride.

N. crassa CBH-1 (NCU07340), GH6-2 (NCU09680), GH5-1 (NCU00762), and GH3-4 (NCU04952) were purified as previously described (*1, 6*). *N. crassa* CDH-1 was partially purified from wild-type *N. crassa* following growth on cellulose. Phosphoric acid swollen cellulose (PASC) was then added (20 mL) to the culture filtrate at 4 °C and mixed for 10 minutes. The PASC was then removed from the culture filtrate by centrifugation for 10 minutes at 4,000 RPM. Next 724 g/L ammonium sulfate was added to the culture filtrate and stirred at 4 °C for one hour. The precipitated proteins were then isolated by centrifugation at 8,000 RPM for 25 minutes. The pellet was resuspended in a minimal volume of water and then buffer exchanged into 25 mM TRIS pH 8.5 using a HiPrep 26/10 desalting column (GE Healthcare). The buffer exchanged proteins were then loaded onto a 10/100 GL MonoQ column and eluted with a linear gradient from 0 to

500 mM sodium chloride. CDH-1 elutes from the column between 50 and 100 mM sodium chloride and is ~80% pure by SDS-PAGE.

N. crassa PMO proteins were purified from the *N. crassa* Δ*cdh-1* strain. Culture filtrates from multiple flasks were pooled and concentrated 100-fold using a tangential flow filtration system with a 5,000 MWCO PES membrane (Millipore). The concentrated culture filtrate was then buffer exchanged into 10 mM Tris pH 8.5 using a HiPrep 26/10 desalting column. The concentrated and buffer exchanged protein was then fractionated using an AKTAexplorer FPLC system (GE Healthcare) and a 10/100 GL MonoQ column. The mobile phases for the anion-exchange fractionation were buffer A: 10 mM Tris pH 8.5 and buffer B: 10 mM Tris pH 8.5 with 1.0 M sodium chloride. For each run on the MonoQ column approximately 100 mg of total secretome protein was loaded and eluted from the column with a linear gradient from 0 to 50% buffer B over six column volumes. NCU08760 and NCU01050 do not bind the column under these conditions and are present in the flow through. NCU02240 and NCU07898 elute from the column between 4-9 mS/cm. Fractions containing the target proteins were pooled and treated with 1.0 mM EDTA overnight to strip bound metals. The EDTA treated samples were then concentrated using 3,000 MWCO PES spin concentrators and desalted into 10 mM Tris pH 8.5 using a 26/10 desalting column. Removal of the bound metal ion increases the affinity of the PMOs for the anion-exchange resin and causes them to elute at higher salt concentrations. For NCU08760 and NCU01050, removal of bound metal ion causes the proteins to bind to the MonoQ column. The apo NCU01050 and NCU08760 are then eluted from the column using the same buffers as above with a linear gradient from 0 to 3.5% buffer B over 3 column volumes. NCU01050 elutes at ~1.6

mS/cm and NCU08760 at ~2.2 mS/cm conductivity. The apo forms of NCU02240 and NCU07898 elute from the MonoQ column between 7-9 mS/cm. The purity of the PMOs can be further improved by reconstituting the apo PMOs with Zn(II) and repeating the anion-exchange fractionation. Other proteins in the secretome do not change their affinity for the resin in the presence or absence of metal ions and can be effectively removed from PMOs by cycles of stripping the metal, fractionating, reconstituting, and fractionating again. NCU01050 and NCU08760 can then be separated from one another by size exclusion chromatography using a Sephacryl S100 column with a mobile phase of 10 mM Tris pH 8.5 with 150 mM sodium chloride. NCU02240 and NCU07898 are separated from one another using the same method as used for NCU01050 and NCU08760. The four natively purified PMOs from *N. crassa* are stable for several months at 4 °C.

CDH binding to Avicel®. Purified CDH-1 or CDH-2 (20 µg) was added to 1.0 mL of 10 mg/mL Avicel® in 50 mM sodium acetate buffer pH 5.0. After incubation for 5 minutes at room temperature, the Avicel® was spun down by centrifugation at 10,000 RPM. The supernatant was removed and the Avicel® pellet was washed with 1.0 mL of fresh 50 mM sodium acetate buffer pH 5.0. The washing step was repeated three times and then the Avicel® pellet was boiled in SDS loading buffer. After boiling, the Avicel® was spun down and the supernatant loaded onto a 10% Tris-HCl polyacrylamide gel.

Metal chelating reaction. To determine the requirement for small molecules and metals, wild-type or Δ*cdh-1* culture filtrate was buffer exchanged more than 10,000-fold in a 10 kDa MWCO PES spin concentrator (Sartorius). The culture filtrate was incubated with

100 μM EDTA for 2 hours. The culture filtrate was then assayed as described above in the presence or absence of CDH-1.

Anaerobic assays. Anaerobic cellulase assays were performed as above except all assays were conducted in an anaerobic chamber (Coy) at room temperature. Buffers were sparged with ultra high purity argon (Praxair, UN 1006) for 1 hour and all enzymes were concentrated to volumes of less than 300 μL before introduction into the anaerobic chamber. All solutions were left open in the anaerobic chamber, and buffer solutions were stirred vigorously for 72 hours before use to fully remove dissolved oxygen. Aerobic reactions were prepared in the anaerobic chamber in 3 mL Reacti-Vials and then removed from the anaerobic chamber, exposed to air, sealed, and returned to the anaerobic chamber. After 24 hours of reaction, assays were centrifuged in the glove bag and 100 μL of assay mix was removed for analysis by the glucose oxidase/peroxidase assay or HPLC and LC-MS.

Screen to identify PMO proteins. The *Δcdh-1* culture filtrate was fractionated over a 10/100 GL MonoQ column as described above. An appropriate concentration of the load, flow-through, and all fractions were added to a mixture of 0.03 mg/mL purified *N. crassa* cellulases (CBH-1, GH6-2, and GH3-4) and assayed on Avicel® for 12 hours. Fractions that had increased cellulase activity in the presence of CDH were kept and analyzed by SDS PAGE and LC-MS/MS.

Protein identification by LC-MS/MS. The identity of the natively purified proteins was confirmed by in-solution tryptic digests and LC-MS/MS of the peptides as previously described (*2*).

Analysis of copper and zinc in the secretome by ICP-AES

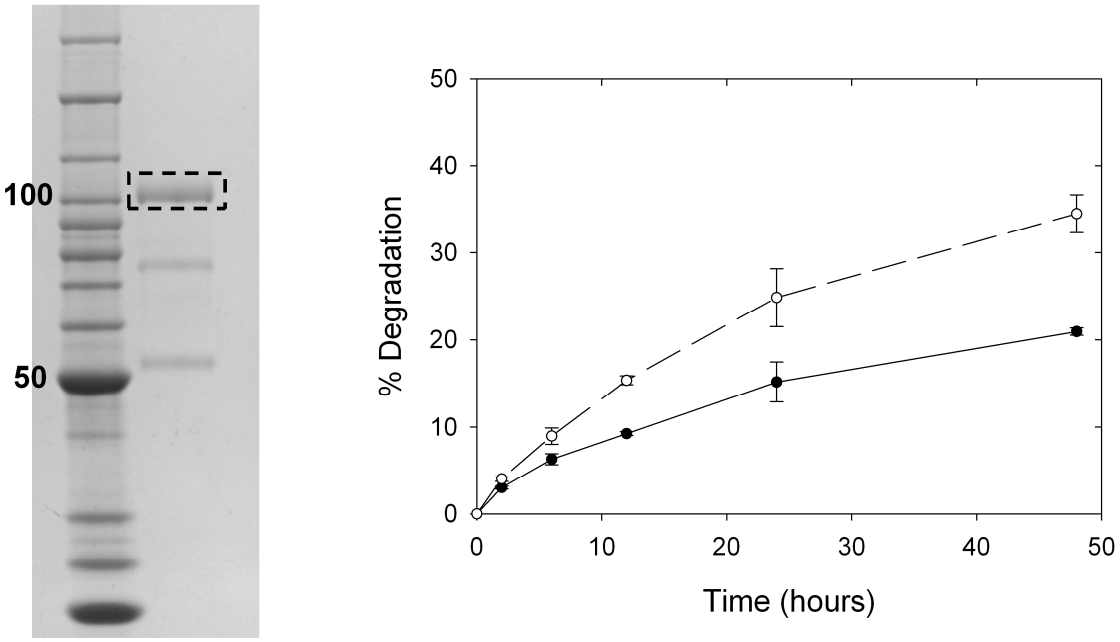
The *N. crassa Δcdh-1* strain was grown on 2% w/v Avicel® as previously described with an additional 5 μM copper sulfate, and 30 μM zinc sulfate supplemented in the minimal media. The culture filtrate was concentrated using tangential flow filtration and buffer exchanged into 10 mM Tris pH 8.5. The concentrated and buffer exchanged culture filtrate was loaded onto a 10/100 GL MonoQ column and separated into 5 fractions with a linear salt gradient. Each fraction was then analyzed for the presence of copper or zinc. Metal analysis was performed using a Perkin Elmer inductively coupled plasma atomic emission spectrometer (ICP-AES) The wavelengths used for quantification for copper were 327.393 or 324.752 nm, and for zinc, 213.857 or 206.200 nm. Samples were also checked for the presence of magnesium, calcium, manganese, iron, and cobalt, none of which were enriched in the concentrated culture filtrate relative to flow through from the spin concentrators.

SUPPLEMENTAL REFERENCES

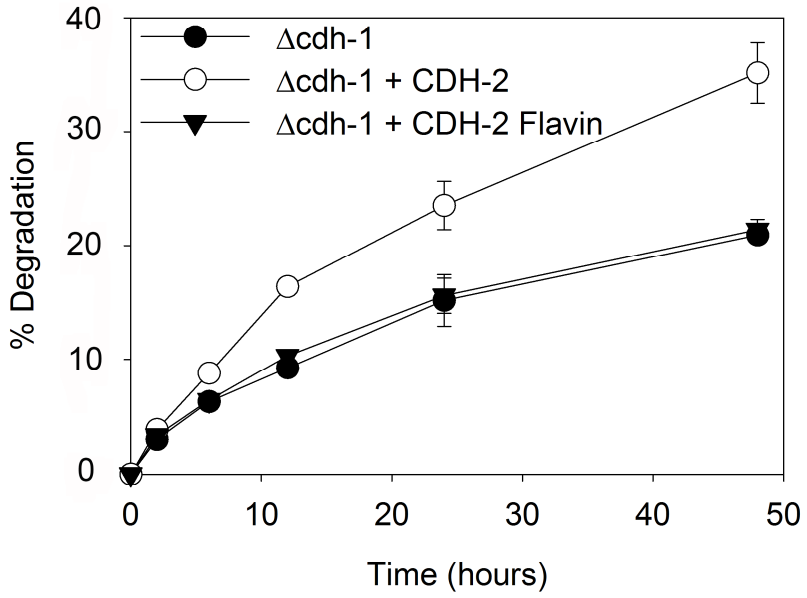
1. Phillips, C. M., Iavarone, A. T., and Marletta, M. A. (2011) Quantitative Proteomic Approach for Cellulose Degradation by Neurospora crassa, *J Proteome Res*, In press.
2. Tian, C., Beeson, W. T., Iavarone, A. T., Sun, J., Marletta, M. A., Cate, J. H., and Glass, N. L. (2009) Systems analysis of plant cell wall degradation by the model filamentous fungus *Neurospora crassa*, *Proc Natl Acad Sci U S A 106*, 22157-22162.
3. Notredame, C., Higgins, D. G., and Heringa, J. (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment, *Journal of Molecular Biology 302*, 205-217.
4. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M., and Gascuel, O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist, *Nucleic Acids Res 36*, 465-469.

5. Canevascini, G., Borer, P., and Dreyer, J. L. (1991) Cellobiose dehydrogenases of *Sporotrichum (Chrysosporium) thermophile*, *Eur J Biochem 198*, 43-52.
6. Sun, J., Phillips, C. M., Anderson, C. T., Beeson, W. T., Marletta, M. A., and Glass, N. L. (2011) Expression and characterization of the *Neurospora crassa* endoglucanase GH5-1, *Protein Expr Purif 75*, 147-154.

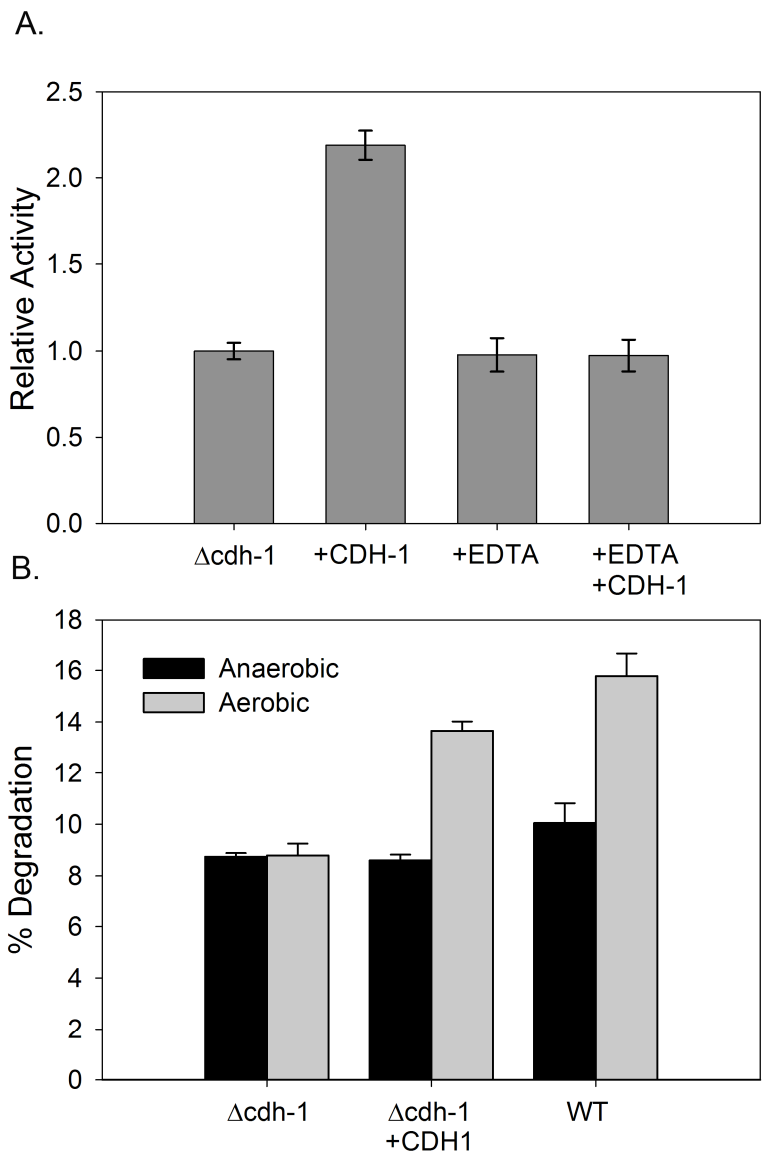
SUPPLEMENTAL FIGURES



Supp. Figure 1. Stimulation of cellulose degradation by the addition of partially purified *N. crassa* CDH-1 to the Δ*cdh-1* culture filtrate. (A) SDS-PAGE of partially purified *N. crassa* CDH-1. (B) Cellulase activity of the Δ*cdh-1* culture filtrate. (□) Represent experiments where 400 µg *N. crassa* CDH-1 per gram of cellulose was added. (•) Represent experiments where no exogenous CDH was added. Values are the mean of three replicates. Error bars are the SD between these replicates.

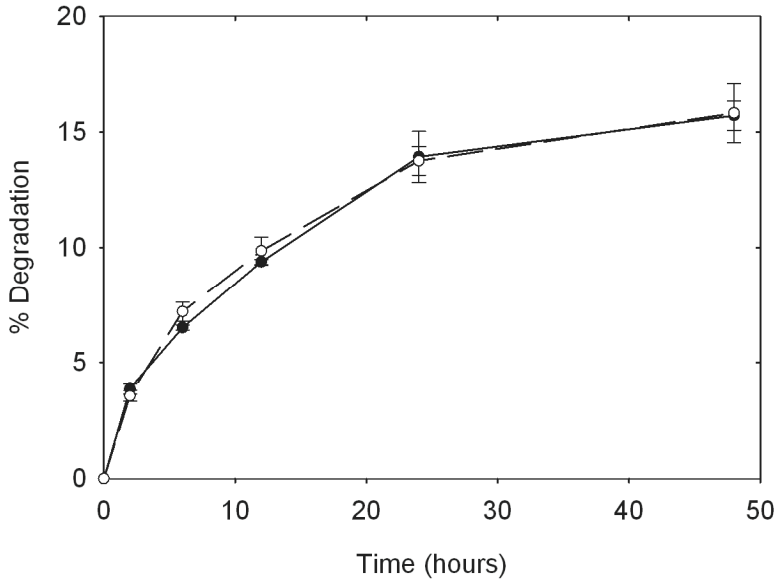


Supp. Figure 2. CDH flavin domain. Stimulation of cellulose degrading capacity of the Δ*cdh-1* culture filtrate by addition of full length CDH-2 (O) or the CDH-2 flavin domain (▼) to the *Δ-cdh-1* culture filtrate (•). Values are the mean of three replicates. Error bars are the SD between these replicates.

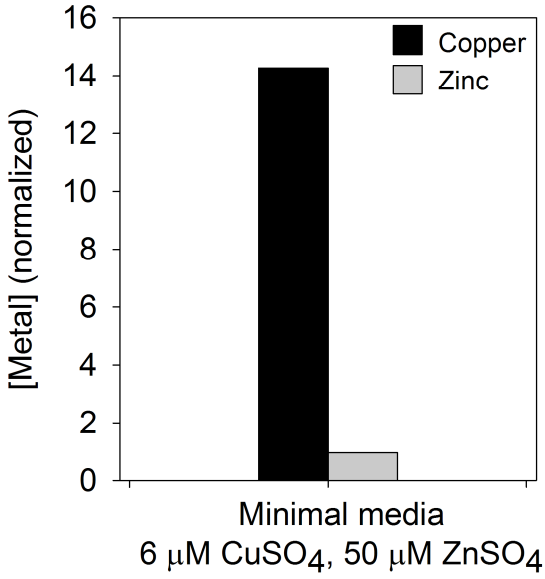


Supp. Figure 3. Contribution of metals or small molecules to the CDH dependent stimulation. (A) Cellulase activity of 10,000-fold buffer exchanged Δ*cdh-1* secretome in the presence or absence of 0.004 mg/mL CDH-1. For reactions containing EDTA, 100 µM EDTA was preincubated with the sample for 2 hours and then assayed. (B) Oxygen dependence of the stimulation of cellulase activity by CDH. Black bars indicate experiments conducted anaerobically, gray bars indicate experiments conducted

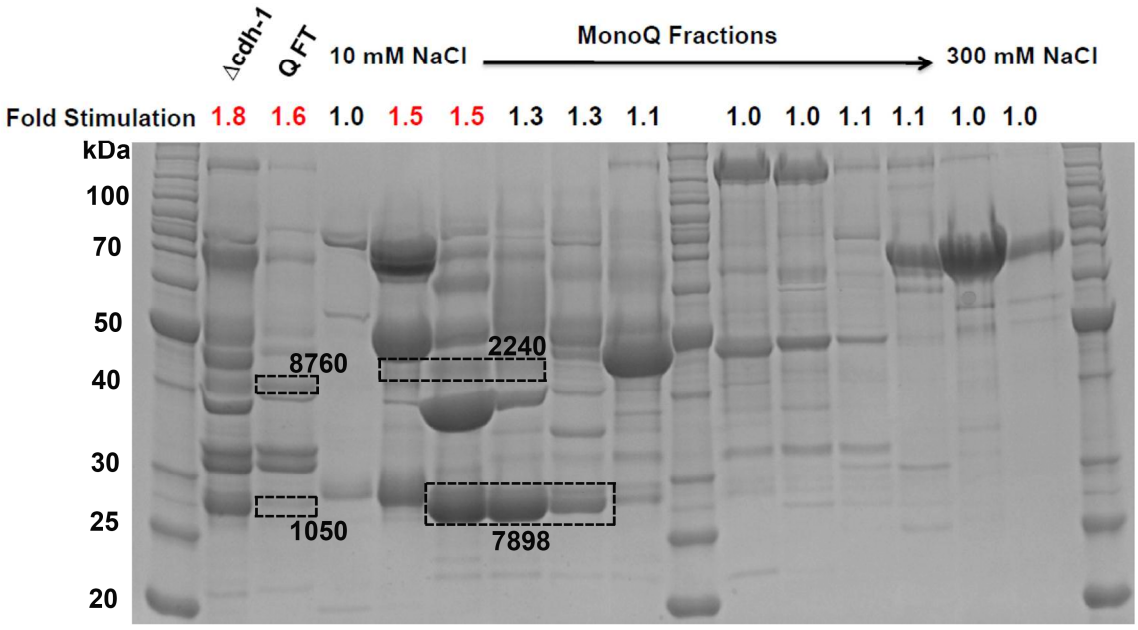
aerobically. Values are the mean of three replicates. Error bars are the SD between these replicates.



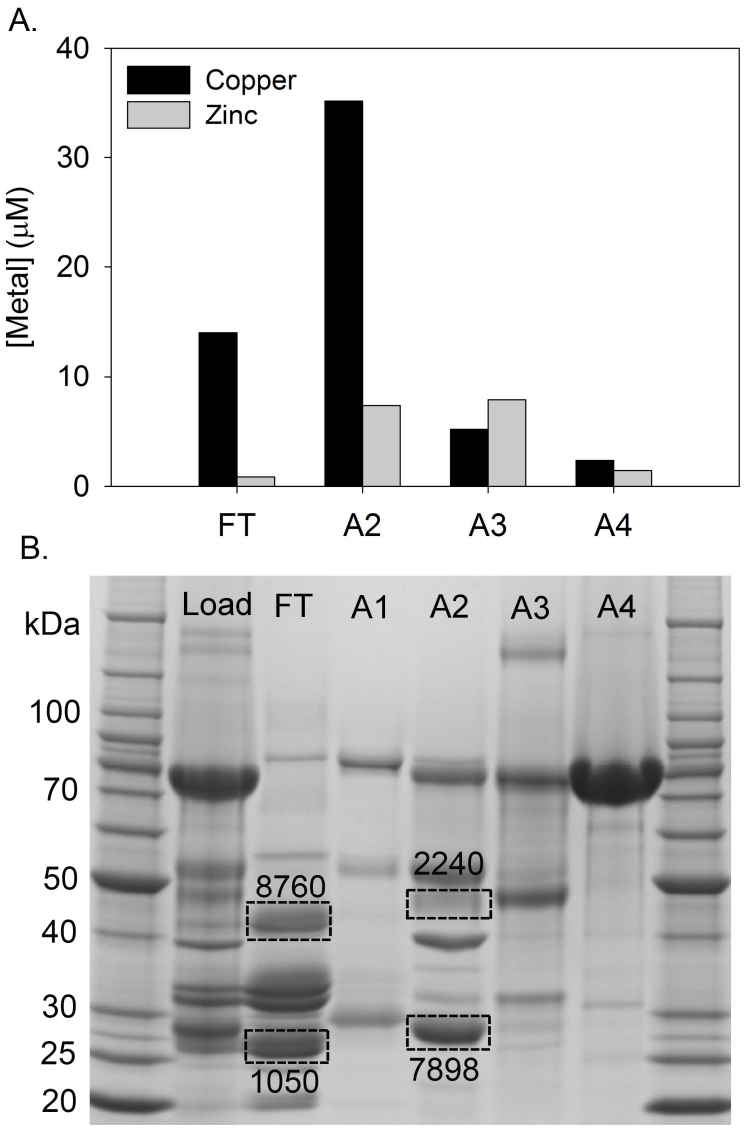
Supp. Figure 4. CDH-1 Addition to Purified Cellulases. *M. thermophila* CDH-1 was added to a mixture of purified cellulases (CBH-1, GH6-2, GH5-1, GH3-4) from *N. crassa* and assayed on cellulose. (●) No exogenous CDH added (○) 400 µg *M. thermophila* CDH-1 per gram of Avicel® added. Values are the mean of three replicates. Error bars are the SD between these replicates.



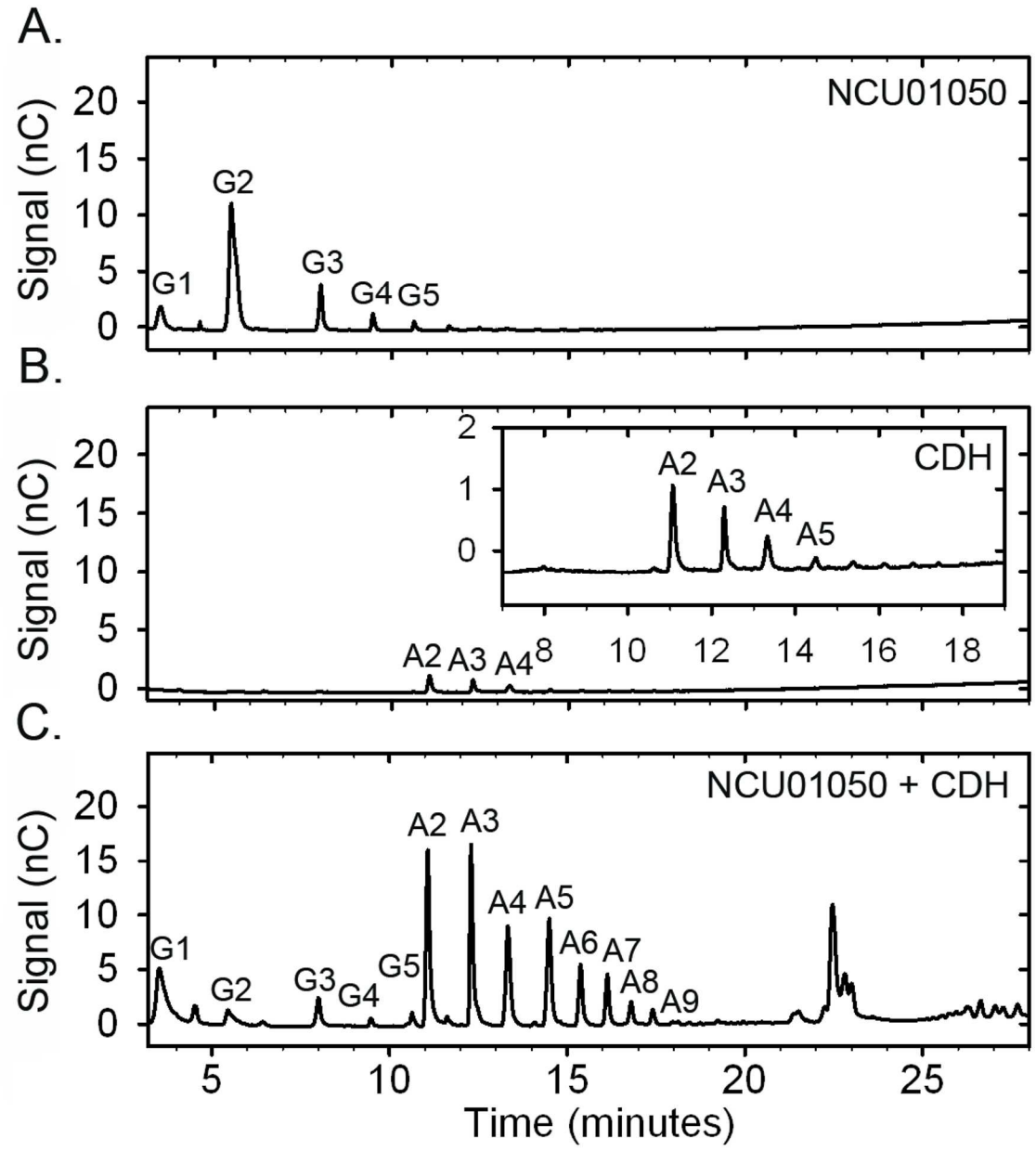
Supp. Fig. 5. ICP-AES metal analysis of the Δ*cdh-1* secretome. Minimal media was supplemented to 6 μM of $CuSO_4$ and 50 μM $ZnSO_4$ and after seven days of growth on Avicel® the fungal biomass was removed by filtration. The secretome was concentrated using 3,000 MWCO spin concentrators and then buffer exchanged into 10 mM TRIS pH 8.5 using a 26/10 desalting column. The micromoles of metal were then normalized to the total protein concentration in the secretome as determined by the Biorad protein assay.



Supp. Fig. 6. Fractionation of the Δ*cdh-1* secretome. A screen was designed to identify other factors involved in CDH dependent enhancement of cellulase activity. Shown is an SDS-PAGE analysis of fractions from MonoQ separation of the Δ*cdh-1* secretome. Above each lane is the fold stimulation of the fraction when added to a mixture of purified *N. crassa* cellulases in the presence or absence of CDH-1. Fractions highlighted in red were analyzed by mass spectrometry and showed the presence of four glycosyl hydrolase family 61 proteins.

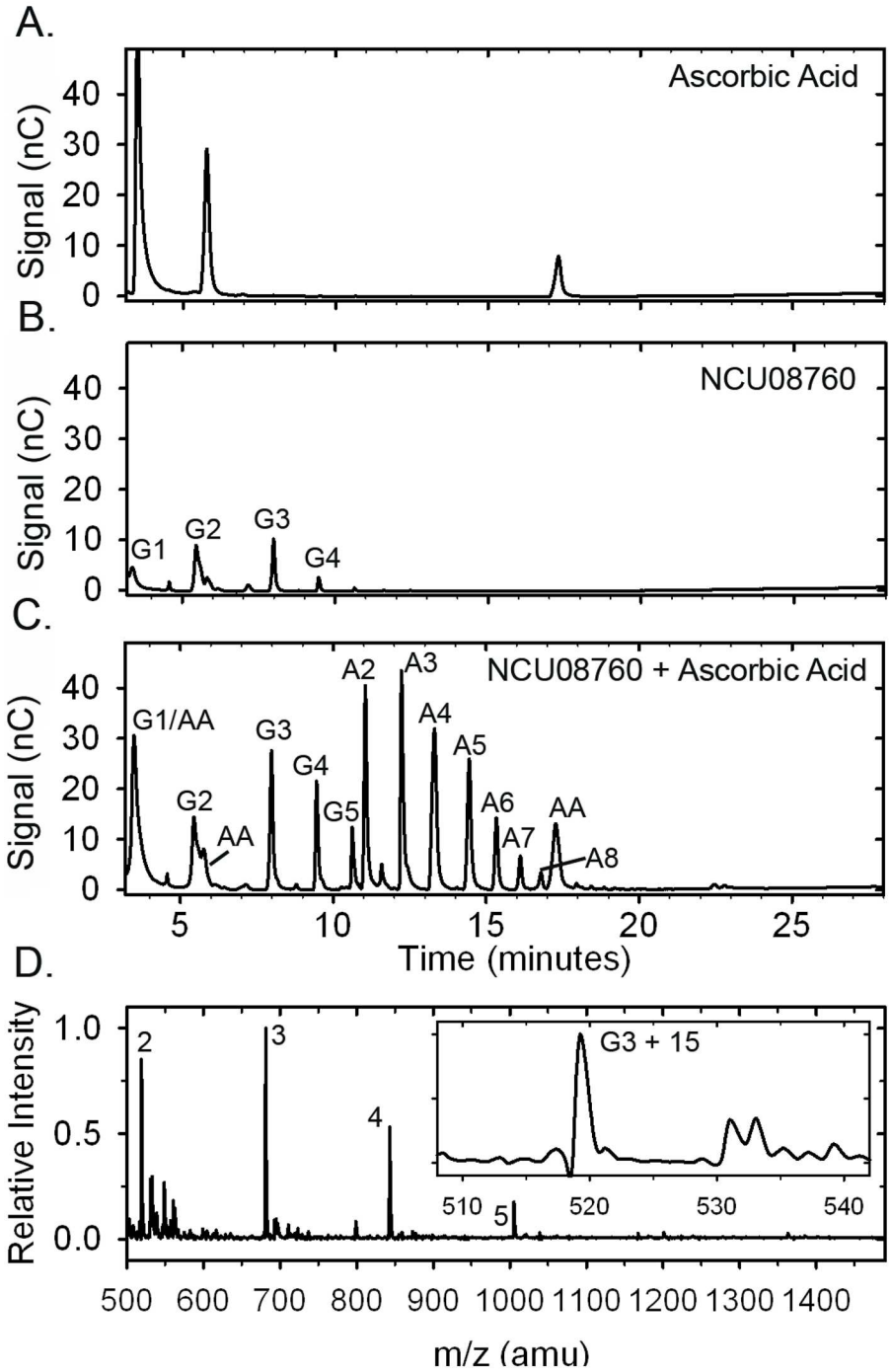


Supp. Fig. 7. ICP-AES metal analysis of MonoQ fractions from the Δ*cdh-1* secretome. The secretome of the Δ*cdh-1* strain was desalted and fractionated with a linear gradient of sodium chloride and analyzed by ICP-AES. (A) Concentrations of copper and zinc in fractions from the MonoQ column. (B) SDS-PAGE analysis of the MonoQ fractions analyzed by ICP-AES.



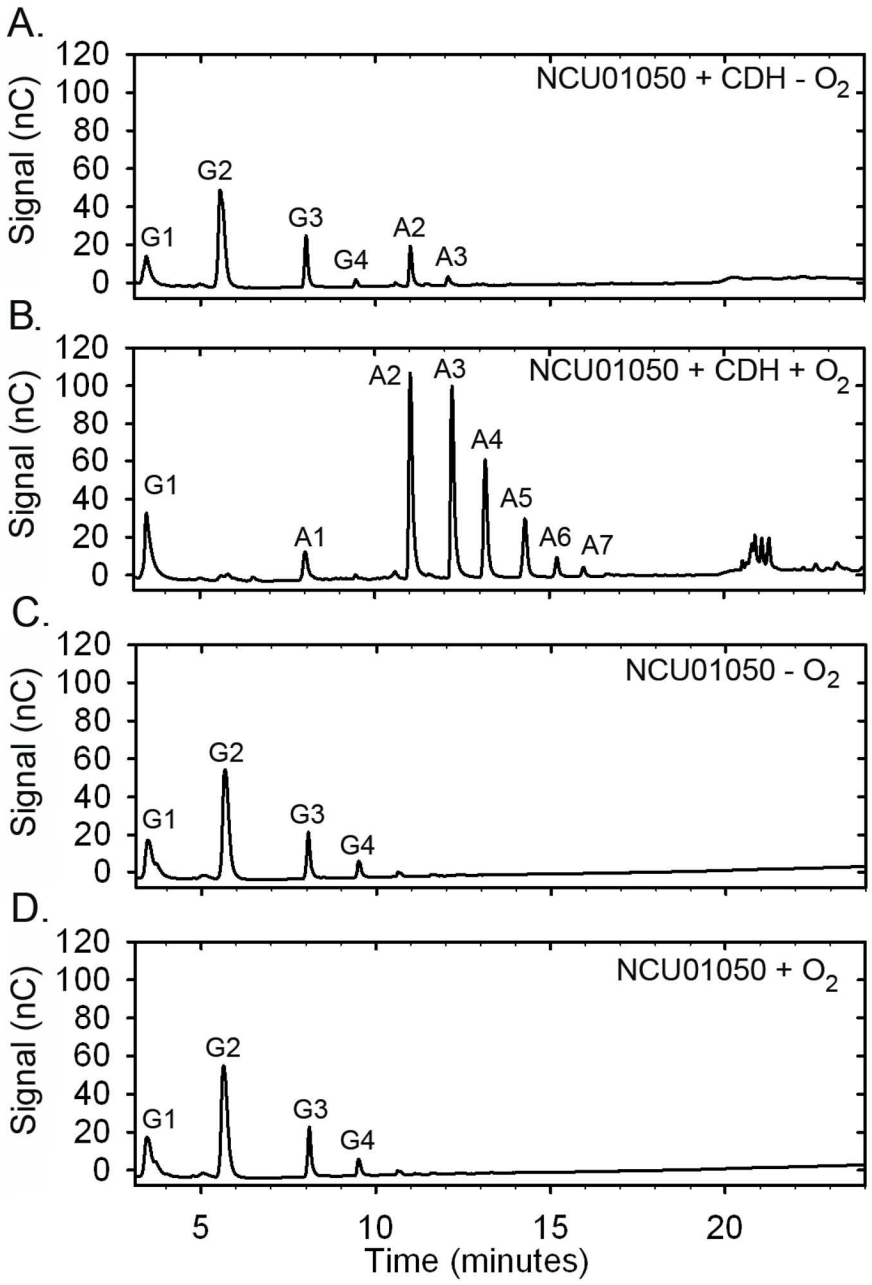
Supp. Fig. 8. HPAEC analysis of CDH-2 and NCU01050 products. (A) GH61 (5.0 µM) (NCU01050), (B) 0.5 µM CDH-2, or (C) a combination of the two were incubated with 5 mg/mL phosphoric acid swollen cellulose (PASC) for 2 hours in 10 mM ammonium acetate pH 5.0 at 40°C. Products were analyzed by HPAEC and standards were used as references to label cello-oligosaccharides (G1-G5) and the respective

aldonic acid derivatives (A2-A9). Inset in (B) indicates the presence of low abundance aldonic acids following treatment with CDH-2 alone. Peaks with retention times of 22-25 minutes correlate with the presence of the Gx + 13 and Gx + 31 species in the LC-MS.



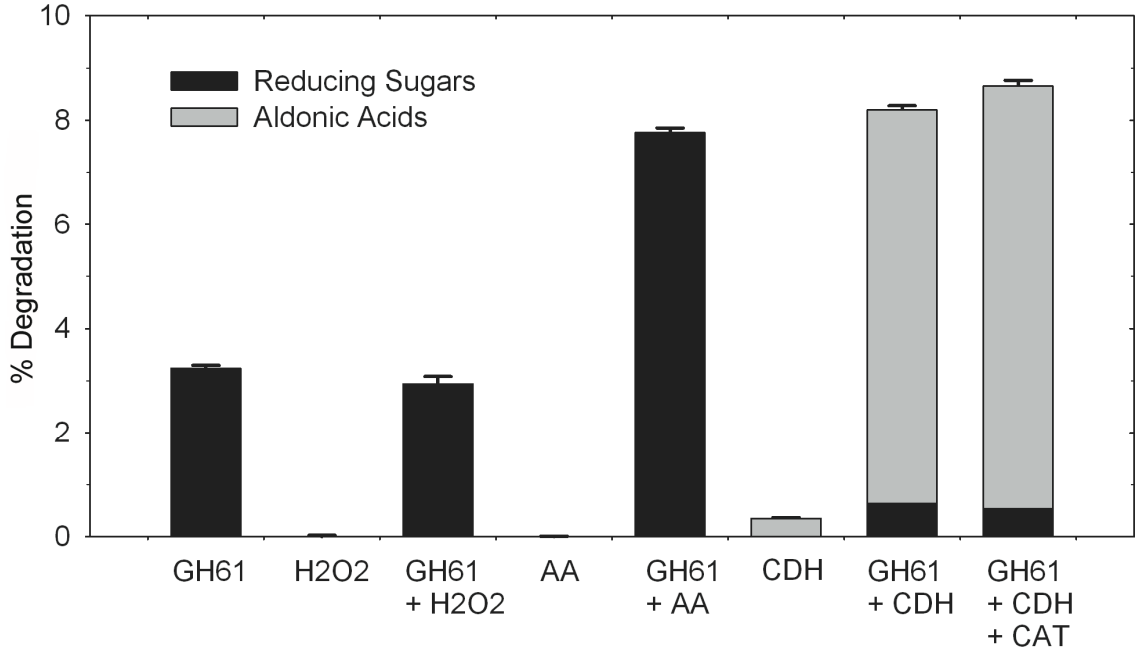
Supp. Fig. 9. HPAEC and LC-MS analysis of NCU08760 products. Ascorbic acid (2 mM), 5.0 µM GH61 (NCU08760) or a combination of the two were incubated with 5 mg/mL phosphoric acid swollen cellulose (PASC) for 2 hours in 10 mM ammonium

acetate pH 5.0 at 40°C. (A-C) Products were analyzed by HPAEC and standards were used as references to label cello-oligosaccharides (G1-G5) and the aldonic acid derivatives (A2-A8). Peaks in the sample containing ascorbic acid only were also present in control reactions where no PASC was present. (D) LC-MS of the reaction containing both ascorbic acid and NCU08760 confirms the production of masses consistent with an aldonic acid. Inset is a zoom around the G3 series.



Supp. Fig. 10. Oxygen dependence of GH61 activity. GH61 (5.0 µM) (NCU01050) and 0.5 µM CDH-2 were incubated with PASC for 24 hours in 10 mM ammonium acetate pH 5.0 under aerobic (A) or anaerobic (B) conditions. In the absence of oxygen, the aldonic acid peaks are significantly reduced in size. Further, the late eluting peak that was consistent with a ladder of 4-keto-aldonic acids is completely absent under anaerobic

conditions. The hydrolytic activity observed by NCU01050 alone was not affected by the presence (C) or absence (D) of oxygen.



Supp. Fig. 11. GH61 cannot be shunted with hydrogen peroxide. GH61 (5.0 µM) (NCU01050) was assayed alone, with 2 mM peroxide, 2 mM ascorbic acid, or 0.5 µM CDH-2. Assays were performed on 5 mg/mL PASC in 10 mM ammonium acetate pH 5.0 with a 24-hour endpoint. Assays containing catalase (*A. niger*) were supplemented with 0.01 mg/mL enzyme. Percent degradation was calculated based on the HPAEC chromatogram and data is shown as a stacked bar graph. Values are the mean of three replicates. Error bars are the SD between these replicates.